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6-Hydroxynicotinic Acid as an Intermediate in the Oxidation of Nicotinic Acid by *Pseudomonas fluorescens*

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As shown by Allinson (1943), many strains of *Pseudomonas fluorescens* can grow on a medium containing inorganic salts with nicotinic acid as a sole source of carbon and nitrogen. When thus grown, washed cell suspensions will oxidize nicotinic acid forming carbon dioxide and ammonia, amongst other end products. The formation of the oxidative system is adaptive (Koser & Baird, 1944; Nichol & Michaelis, 1947). More recent studies (Pinsky & Michaelis, 1952) suggest that the pyridine ring is split during the first stages of the oxidative attack and that either carbon 2′ or carbon 6′ is involved in the ring-opening reaction. In the present paper two lines of approach were used to study the oxidation. First, the oxidation of some halogen-substituted nicotinic acids was studied; the results indicated that carbon 6′ rather than carbon 2′ was involved in the ring-opening reaction. Secondly, the possibility that intermediates in the oxidation might accumulate during adaptation was examined and 6-hydroxynicotinic acid was isolated in the early stages.

EXPERIMENTAL

Organisms. Three strains of *Ps. fluorescens* were isolated from soil-enrichment cultures by Mrs M. Kogut, Microbiology Department, Sheffield (Kogut & Brodoski, 1953). The strain (KBI) used in most of the experiments was isolated from medium containing 0·1% sodium benzoate as a sole source of carbon; strain 221 was isolated from a citrate medium, and strain 224 from a succinate medium. The three strains grew readily in the inorganic medium of Koser & Baird (1944) containing 0·1% nicotinic acid as a sole source of carbon and nitrogen; cells thus grown are referred to as 'adapted cells'. Cells not grown with nicotinic acid are referred to as 'non-adapted cells' and were grown on the inorganic medium of Koser & Baird with 0·1% asparagine. The cultures were maintained by subculturing twice-weekly upon solid medium containing 1·0% yeast autolysate, 1·0% asparagine and 2·5% agar; cultures were incubated at 25°.

Preparation and handling of washed suspensions. Cells for manometric experiments were grown in 200 ml. batches contained in 1 l. penicillin culture flasks which were shaken at room temperature for 18–24 hr. Each flask was inoculated with a loop of the culture from the solid medium. The average yield of cells was 100–150 mg. dry wt./200 ml. medium. After growth, the cells were collected by centrifuging, washed twice and then suspended in 0·9% NaCl to give a suspension containing 10–12 mg. dry wt./ml. Dry wts. were estimated by drying a sample at 110° and subtracting the dry wt. of an equal vol. of 0·9% NaCl. In some experiments cells were grown on the surface of a medium solidified by the addition of 2·0% agar.

The oxidation of nicotinic acid was followed manometrically in a Warburg apparatus. Each flask contained cell suspension (1·0 ml.) and 0·1 M phosphate buffer (KH_2PO_4 adjusted with N-NaOH), pH 7·0 (0·5 ml.) in the main compartment. Nicotinic acid (0·5 ml.) was added from the side arm after equilibration for 20–30 min. at 30°. The centre well contained folded paper and 2N-KOH (0·2 ml.) to absorb CO_2. Rates of oxidation are expressed as μmoles O_2 absorbed/mg. dry wt. cells/hr. Both the rate and uptake were calculated by subtracting the O_2 uptake of a blank experiment with water instead of substrate. The value of the blank is also stated in most experiments.

Materials. The nicotinic acid used in preliminary studies was a specimen used as an analytical standard in the laboratory. It had been sublimed at 230° and later recrystallized from aqueous ethanol (Knight, 1937; Hughes & Williamson, 1952). This was assumed to be pure until it was found that immediately upon its addition to non-adapted cells there was an O_2 uptake of 0·25–0·5 μmole O_2/μmole nicotinic acid added/20 min. but no corresponding

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disappearance of nicotinic acid. In view of similar findings by Lindgren & Palleroni (1952) with galactose this immediate oxygen uptake was assumed to be due to some contaminant rather than to any 'preadaptive oxidation' (Reiner & Spiegelman, 1947). Another batch of nicotinic acid (British Drug Houses Ltd.) was found to behave similarly, whilst a third batch (Light and Co. Ltd.) inhibited both growth and adaptation, as compared with the other two. The three batches were purified by treatment with Norite charcoal in water at 80° followed by crystallization, sublimation at 230° and recrystallization from water (see Hughes & Williamson, 1953). No 'preadaptive' O_2 uptake was then found with any of the specimens. The halogen-substituted nicotinic acids were purified as previously described (Hughes, 1954). 6-Hydroxynicotinic acid was a commercial specimen (Light and Co. Ltd.) purified by treatment with Norite and crystallization from water; 2-hydroxynicotinic acid was prepared by the method of Phillips (1895); α-hydroxypyridine was a commercial specimen (Light and Co. Ltd.) and was not further purified; O_2/mg. dry wt. cells/hr. The total consumption was 3·6–4·3 μmoles O_2/μmole nicotinic acid and is in agreement with the results of Pinsky & Michaelis (1952), but higher than those of Koser & Baird (1944). 2:4-Dinitrophenol (10^{-2}–10^{-4}M) had no effect on oxygen consumption. The average rate of oxidation of 5-fluoronicotinic acid (5-FNA) was 10–20 % lower than that of nicotinic acid (Table 1). In the same batch of cells the total oxygen consumption for the oxidation of 5-FNA was less (0·2–0·5 mole O_2/mole substrate) than that consumed in the oxidation of nicotinic acid (Table 1).

After the oxygen uptake in the presence of nicotinic acid and 5-FNA had ceased, the contents of the flasks were placed in a boiling-water bath for 5 min. and the solid material was removed by centrifuging. A sample of the clear supernatant (0·1 ml.) was placed on a paper chromatogram in a spot measuring

Table 1. *The oxidation of nicotinic acid and derivatives by washed suspensions of Ps. fluorescens*

Cells were grown on the liquid salt medium containing 0·1 % nicotinic acid; after washing they were added to Warburg flasks (3·8–10 mg. dry wt.) together with substrate (2·0–5·0 μmoles). The rate of endogenous O_2 uptake (0·3–1·0 μmole O_2/mg. dry wt./hr.) has been allowed for in all experiments.

Substrate	No. of expts.	Total oxygen uptake (μmoles O_2/μmole substrate added) Range	Mean	Rate of oxygen uptake (μmoles O_2/mg. dry wt. cells/hr.)
Nicotinic acid	11	3·6–4·3	3·9	3·1–7·2
5-Fluoronicotinic acid	7	3·5–4·0	3·8	2·2–6·0
5-Chloronicotinic acid	6	1·3–2·3	1·9	1·0–4·5
2-Fluoronicotinic acid	7	0·7–1·5	1·1	1·3–6·5
6-Fluoronicotinic acid	6	0·0–0·45	0·1	0·2–0·3
6-Hydroxynicotinic acid	3	3·0–3·6	3·3	2·0–4·0

2:6-dihydroxynicotinic acid was prepared by the method of Guthzeit & Laska (1898).

Chemical estimations. Ammonia was estimated by distillation in a modified Parnas apparatus followed by nesslerization. Nicotinic acid was estimated by the modified König reaction described by Hughes & Williamson (1953). Nicotinic acid and other pyridine derivatives were detected on paper chromatograms by examination in ultraviolet light and were photographed as described by Markham & Smith (1949). The CNBr spray reagent (Kodicek & Reddi, 1951) was used to detect pyridine derivatives not substituted in the α carbon. The solvents used for developing paper chromatograms were: *n*-butanol–acetic acid–water (Partridge & Westall, 1948); propanol–ammonia–water (Hanes & Isherwood, 1949); *n*-butanol–ethylamine (Hiscox & Berridge, 1950); ethyl acetate–water (Wollish, Schmall & Schaffer, 1951). Spectrophotometric measurements were made in a Unicam spectrophotometer.

RESULTS

Oxidation of nicotinic acid and halogen-substituted nicotinic acids by adapted cells

Washed suspensions of strain KBI, grown for 18–24 hr. on the nicotinic acid medium, oxidized nicotinic acid (2–5 μmoles) at a rate of 3–7 μmoles about 2 × 1 cm. and the chromatograms were developed as described. No detectable products of oxidation were found when the chromatograms were examined in ultraviolet light, by spraying with the CNBr reagent of Kodicek & Reddi (1951) or with the bromocresol green spray for organic acids (Buch, Montgomery & Porter, 1952).

The rate of oxidation of 5-chloronicotinic acid (5-ClNA) was 20–30 % that of nicotinic acid and proceeded linearly for 80–140 min. Oxygen uptake ceased when 1·3–2·3 μmoles of oxygen had been taken up for each μmole of 5-ClNA added. Tests with the CNBr reagent showed that at least 50 % of the substrate was still able to react and was thus unaltered in the 6′ or 2′ carbon position. Examination of the mother liquors by paper chromatography showed a product absorbing ultraviolet light, but not reacting with CNBr reagent. This product, when eluted from the paper and dissolved in N-NaOH or N-HCl, had an absorption spectrum typical of a pyridine derivative (Fig. 2), suggesting that a substitution in the α-carbon had taken place. In view of subsequent experiments with nicotinic acid it is likely that this product was 5-chloro-6-hydroxy-

nicotinic acid. This substance could not be isolated because insufficient amounts of 5-ClNA were available. In most batches of cells 5-bromonicotinic acid was not oxidized at a significant rate. The rate of oxidation of 2-fluoronicotinic acid (2-FNA) was generally the same as that of 5-FNA but the total oxygen consumption was considerably less (0·7–1·5 μmoles O_2/μmole 2-FNA); 6-fluoronicotinic acid was generally not oxidized at a significant rate (Table 1).

The oxidation of nicotinic acid during the period of adaptation

The results on the oxidation of 5-FNA and 5-ClNA described in the previous section supported the suggestion (Pinsky & Michaelis, 1952) that an early step in the oxidation of nicotinic acid is an opening of the pyridine ring and that the α-carbon is involved. It was not possible, however, to identify any intermediates formed. Studies were therefore continued on the oxidation of nicotinic acid itself and with cells grown on asparagine instead of nicotinic acid. This approach was developed from the following considerations. In the past, the mechanism of the oxidation of the aromatic ring by bacteria has been investigated by two main methods. First, the attempt to isolate the intermediates in the oxidation from the products of growing cultures has been used mainly by Happold & Evans (see Happold 1950); secondly, the application of the technique of 'simultaneous' or 'successive' adaptation has been put to wide use by Stanier (1947). This second method relies on the fact that each step in the oxidation of a substrate is controlled by at least one specific enzyme and that these enzymes are formed or otherwise became active successively, during adaptation. As judged by the length of time taken for cells to adapt to intermediates in an oxidative series, it seems likely that some enzymes are formed more easily than others (Stanier, 1947); it was thought, therefore, that if such were the case during adaptation to nicotinic acid, then during the adaptive period some intermediate might accumulate where the rate of enzyme formation became the rate-limiting reaction. This idea was tested in the present experiments as follows. Cells of strain KBI which had been grown with asparagine as the sole carbon source were collected, washed and suspended in 0·9 % NaCl (10 mg. dry wt./ml.). The suspension (1·0 ml.) was placed in Warburg flasks together with nicotinic acid (0·002 M final concn.) and the volume made to 2·0 ml. with water. The flasks were incubated at 30° and the rate of oxygen uptake followed. Oxygen uptake continued at the same rate as in the controls containing no nicotinic acid for *ca.* 90–120 min., after which there was a successive increase until the maximum rate was assumed (90–140 min.) (Fig. 1). The length of the lag period, i.e. the time before the first increase in rate of uptake, which may be considered as the time taken for the cells to adapt to nicotinic acid, was not affected by the addition of phosphate buffer (0·005–0·05 M) pH 7·0, nor by NH_4Cl (0·1 M). The lag period was however decreased by raising the concentration of nicotinic acid to 0·015 M, but the time taken for the rate of uptake to reach its maximal value was not decreased under these conditions (Fig. 1). This

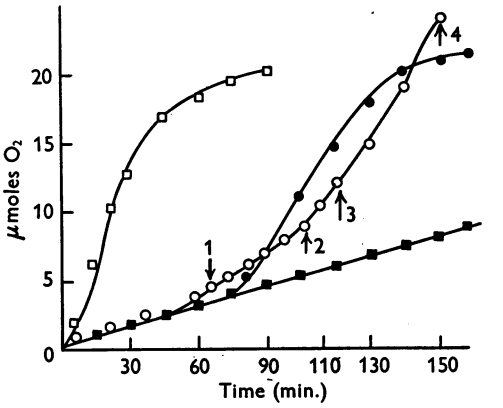

Fig. 1. Oxidation of nicotinic acid by washed suspensions of *Ps. fluorescens*. □, Cells grown on nicotinic acid and with nicotinic acid as substrate (4 μmoles); ■, cells grown upon asparagine with no added substrate; ●, cells grown upon asparagine with nicotinic acid (4 μmoles) as substrate; ○, cells grown upon asparagine with nicotinic acid (30 μmoles) as substrate (flasks removed as indicated by arrows).

meant that the rate of oxygen uptake increased slightly at about 40–60 min. and continued at this slightly faster rate for about 20–30 min. and until from 0·5–0·6 μmole O_2/μmole nicotinic acid had been consumed. It seemed likely that during this early slight increase in rate of oxygen uptake some intermediate was formed to which the cells adapted slowly. Four pairs of Warburg flasks were therefore prepared, each pair containing the suspension of non-adapted cells (1·0 mg. dry wt.), one with nicotinic acid (30 μmoles) and the other water to serve as control. The flasks were incubated at 30° and removed from the bath in pairs as follows: (1) and (2) at the beginning and end of the first increase in rate, (3) and (4) at the beginning and during the period of maximal rate of oxygen uptake (Fig. 1). The contents of the flasks were centrifuged, the supernatants decanted and 0·1 ml. of each placed upon paper chromatograms. The chromatograms were developed in the butanol–acetic acid and butanol–ethylamine solvents, photographed in ultraviolet light and then sprayed with the CNBr reagent for nicotinic acid. The presence of a new

ultraviolet light absorbing spot other than nicotinic acid was detected in mother liquors from flasks (1), (2) and (3) (Fig. 1), that is where the reaction had been stopped during the first period of increased rate of oxygen uptake. None of the new material was detected in the flask removed during the period of maximal uptake. The absorption spectra of solutions of the mother liquors and of the eluted spots in either HCl or NaOH suggested the presence of a pyridine derivative (Fig. 2). It did not react with CNBr, which indicated substitution in the α-carbon position.

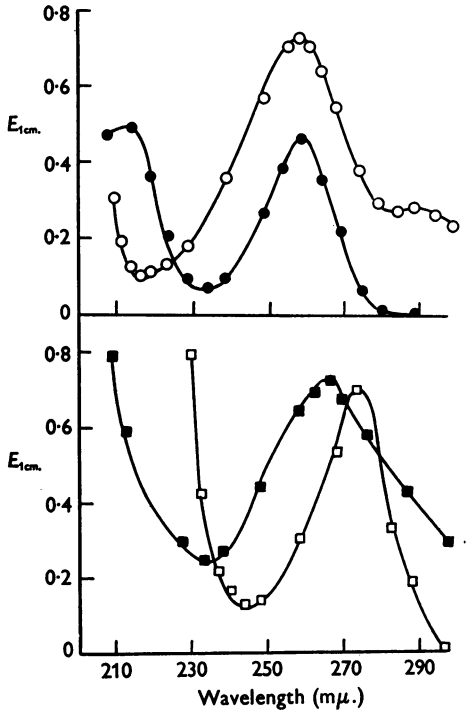

Fig. 2. Absorption spectra of nicotinic acid, 5-chloronicotinic acid and products of their oxidation by *Ps. fluorescens*. All solutions are in N-HCl. ●, Nicotinic acid; ○, mother-liquor during oxidation of nicotinic acid by cells grown upon asparagine; □, 5-chloronicotinic acid; ■, 5-chloronicotinic acid after oxidation.

These results, together with those from the experiments with the halogen analogues, lend further support to the idea that the first reaction in the oxidative attack is one of substitution in carbon 2′ or 6′. Argument by analogy with the established mechanism of the oxidation of the aromatic ring in bacteria (Evans, Smith, Linstead & Elvidge, 1951) suggested that the substituent group might be a hydroxyl and that the new product might be 2- or 6-hydroxynicotinic acid. The chromatographic and spectroscopic properties of 6-hydroxynicotinic acid, 2-hydroxynicotinic acid and α-hydroxypyridine were therefore compared with the new intermediate.

The R_F of the new product in the butanol–acetic acid solvent was 0·70 and in the butanol–ethylamine solvent was 0·25. The corresponding R_F values for nicotinic acid are 0·78 and 0·88, for 6-hydroxynicotinic acid 0·70 and 0·25, and for 2-hydroxynicotinic acid 0·68 and 0·23. Both the new material and 6-hydroxynicotinic acid give ultraviolet absorbing spots on paper whilst 2-hydroxynicotinic acid fluoresces. The absorption spectra in

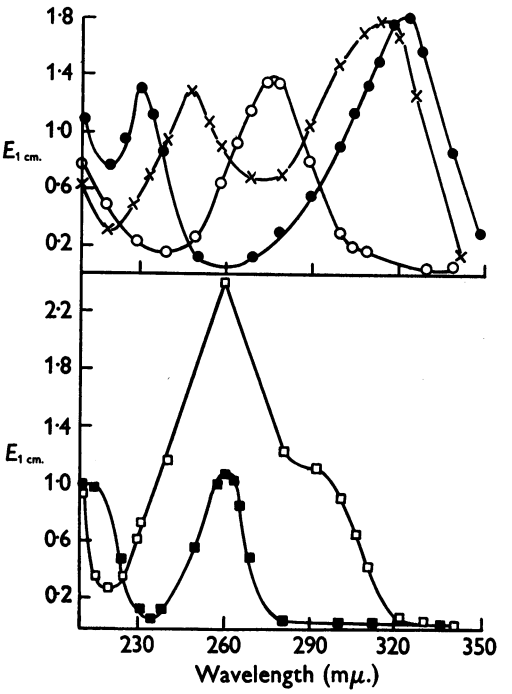

Fig. 3. Absorption spectra of nicotinic acid, hydroxynicotinic acids and α-hydroxypyridine. All solutions were 2×10^{-4} M in respect of the pyridine derivative and in N-HCl. ■, Nicotinic acid; □, 6-hydroxynicotinic acid; ○, α-hydroxypyridine; ●, 2-hydroxynicotinic acid; ×, 2:6-dihydroxy nicotinic acid.

N-HCl of the new material and 6-hydroxynicotinic acid are identical (Figs. 2 and 3). The new product thus resembles 6-hydroxynicotinic acid in chromatographic and spectrographic properties. The identity was established by isolation.

Isolation of 6-hydroxynicotinic acid

Non-adapted cells were grown for 18 hr. on 1 l. of solid asparagine medium contained in three $8 \times 11\frac{1}{2}$ in. enamelled iron trays. After washing, the

cells (440 mg. dry weight) were suspended in 50 ml. 0·1 M phosphate buffer (pH 7·0) to which were added 50 ml. 0·01 M nicotinic acid. A 1 ml. sample was placed in a Warburg flask. Another 1 ml. sample was centrifuged immediately and 0·2 ml. of the clear mother liquor added to 3·0 ml. N-HCl. Both the Warburg flask and the main bulk of the cells, contained in a 500 ml. conical flask, were shaken at 30°. Samples were removed at intervals from the bulk suspension (see Table 2), centrifuged and diluted in N-HCl as above. To detect the formation of 6-hydroxynicotinic acid, advantage was taken of the fact that its optical density at 260 mμ. is much greater than that of nicotinic acid (Figs. 2 and 3). The $E_{260\,m\mu}$ of the mother liquors from successive samples were therefore measured. Shaking of the Warburg flask and main bulk was stopped temporarily during the preparation of sample and measurement of optical density. The optical density showed the expected increase as soon as the rate of oxygen consumption increased (Table 2). The reaction in the bulk of the material was stopped by centrifuging off the cells when $E_{260\,m\mu}$ showed a decrease, indicating that 6-hydroxynicotinic acid was being removed faster than it was being formed (Table 2). Readings were also taken at the isosbestic point (220 mμ.) as a further check on the oxidation of 6-hydroxynicotinic acid.

Table 2. *Oxygen uptake and changes in optical density of mother liquor during adaptation*

For experimental details see p. 306.

Incubation time (min.)	Oxygen uptake (μmoles O_2/mg. dry wt./hr.)	Optical density	
		$E_{280\,m\mu}$	$E_{260\,m\mu}$
0	1·0	1·48	0·092
60	1·4	1·31	0·462
80	3·2	1·2	0·643
100	3·2	0·97	0·88
120	3·7	0·87	1·25
150	4·6	0·83	1·1

After centrifuging, the clear supernatant was decanted, adjusted to just below pH 1·0 with N-HCl and extracted 4 times with half its volume of *n*-butanol saturated with water. The butanol extract was evaporated to dryness *in vacuo* and the residue dried by evaporating twice with absolute ethanol. To remove any nicotinic acid, the gummy residue was extracted with 10 ml. of boiling ethanol and then twice further with cold ethanol. The ethanol-insoluble residue was dissolved in about 25 ml. of hot water, boiled 5 min. with 0·2–0·5 gm. Norite charcoal, filtered, evaporated *in vacuo* to 6–8 ml. and stored at 2° overnight. The slightly coloured crystals which separated were recrystallized from water, washed in cold ethanol and ether and dried over P_2O_5; yield 12 mg., sintered at 304°, melted with gas evolution and decomposition at 314°. An authentic specimen of 6-hydroxynicotinic acid sintered at 304°, melted with gas evolution and decomposition 314–316° and was unchanged by admixture with the isolated material.

The absorption spectra of the crystals and of authentic 6-hydroxynicotinic acid in N-HCl were identical ($\epsilon_{max.\ 260\,m\mu} = 1{\cdot}24 \times 10^4$).

Oxidation of 6-hydroxynicotinic acid by washed suspensions of Pseudomonas fluorescens

According to Stanier's (1947) concept of simultaneous adaptation, it would be expected that if 6-hydroxynicotinic acid were an intermediate in the oxidation of nicotinic acid, then cells adapted to oxidize the latter would also oxidize 6-hydroxynicotinic acid without an appreciable lag phase. Washed suspensions of strain KBI which had been grown on nicotinic acid did in fact oxidize 6-hydroxynicotinic acid at the rate of 6 μmoles O_2/mg. dry wt./hr. and with a final uptake of 3·2–3·8 moles O_2/mole 6-hydroxynicotinic acid (Fig. 4*a*); suspensions of strains 222 and 224 gave similar results. On the other hand, the cells did not oxidize 2-hydroxynicotinic acid, α-hydroxypyridine or 2:6-dihydroxynicotinic acid. Non-adapted cells adapted to oxidize 6-hydroxynicotinic acid (Fig. 4*b*); the length of the lag period was about the same as with nicotinic acid. Cells did not adapt to 2-hydroxynicotinic acid, 2:6-dihydroxynicotinic acid nor to α-hydroxypyridine when incubated for periods up to 4 hr. These results lend additional support to the idea that 6-hydroxynicotinic acid is an intermediate in the oxidation of nicotinic acid.

It is also of additional interest to note that cells adapted to oxidize 6-hydroxynicotinic acid also oxidized nicotinic acid itself without any lag phase (Fig. 4*b*). No nicotinic acid was detected in the 6-hydroxynicotinic acid used in these experiments. It must be assumed therefore that the 6-hydroxy analogue is sufficiently similar to the parent compound to cause adaptation (cf. Cohn & Monod, 1953; Bernheim, 1953).

The effect of halogenonicotinic acids on the growth of and adaptation to nicotinic acid by Ps. fluorescens

The three strains KBI, 221 and 224 grew readily on medium containing 0·05–0·25 % nicotinic acid as a sole source of carbon. None of the strains would grow when nicotinic acid was replaced by 0·02–0·1 % 5-FNA, 2-FNA, 6-FNA, 5-ClNA or 5-BrNA. The addition of the halogen analogues (0·1 %) to medium containing nicotinic acid (0·1 %) did not inhibit growth when the inoculum had been previously grown with nicotinic acid, i.e. was already adapted. Some delay in growth was found when the inoculum was grown on asparagine, i.e. was non-adapted, suggesting that the halogen

analogues might inhibit adaptation to nicotinic acid. Similar results have been subsequently found with halogen-substituted benzoic acids (Hughes, 1953). When tested manometrically, washed suspensions were found to adapt to oxidize 5-FNA but not 2-FNA, 6-FNA, 5-ClNA or 5-BrNA. The rate of adaptation to 5-FNA was much slower than adaptation to nicotinic acid (Fig. 4). The effect of the halogen analogues on adaptation to nicotinic acid by washed suspensions was not tested further.

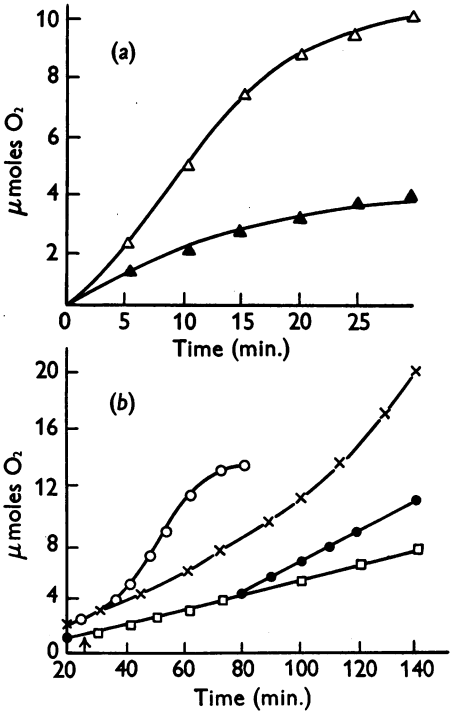

Fig. 4. Oxidation and adaptation to 6-hydroxynicotinic acid and 5-fluoronicotinic acid followed manometrically. (*a*) Cells grown on nicotinic acid; △, 6-hydroxynicotinic acid (2·0 μmoles) as substrate; ▲, no substrate. (*b*) Cells grown on asparagine; ×, 6-hydroxynicotinic acid (50 μmoles) as substrate; ●, 5-fluoronicotinic acid (10 μmoles) as substrate; ○, cells, after adapting to 6-hydroxynicotinic acid, were washed and nicotinic acid (2·0 moles) was added as substrate at the time indicated by the arrow; □, no substrate.

Oxidation of nicotinic acid by cell-free extracts

Previous workers (Pinsky & Michaelis, 1952) were unable to obtain cell-free bacterial extracts which oxidized nicotinic acid. It has now been found that extracts of cells crushed in a bacterial press (Hughes, 1951) readily oxidize nicotinic acid to 6-hydroxynicotinic acid. To prepare the extracts, *Ps. fluorescens* strain KBI was grown on 2 l. of the solid medium containing nicotinic acid, distributed equally between nine enamelled iron trays measuring 10·5 × 14 in. The cultures were incubated for 22 hr. at room temperature, producing relatively young adapted cells. Cells (12·4 g. wet wt.) were washed from the agar with 0·9% NaCl, strained through glass wool, collected by centrifuging and washed twice. After crushing at about −30°, the viscous material was mixed for 5 min. in a metal Potter-type homogenizer with 25 ml. of ice-cold 0·5 M phosphate buffer, pH 7·0, and then centrifuged for 10 min. at 2500 *g*. The cloudy supernatant extract on microscopic examination showed no detectable intact cells. The extract was salmon-pink in colour, highly viscous, and although it pipetted reasonably well it did not mix readily upon dilution. In order to assist dispersion therefore, six glass beads, 4 mm. diam., were added to Warburg flasks which were then shaken with a circular motion before attachment to manometers. All solutions and Warburg flasks were kept in ice water during assembly and the

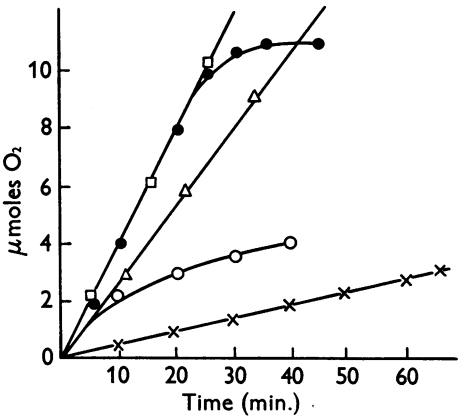

Fig. 5. Oxidation of nicotinic acid by cell-free extracts of *Ps. fluorescens*. Each manometer flask contained cell-free extract (1·0 ml.) and nicotinic acid as indicated; total vol. 2·0 ml. Nicotinic acid concn.: ×, Nil; ○, 5 μmoles; ●, 10 μmoles; □, 50 μmoles; △, 100 μmoles.

contents of the flasks were mixed after a minimal equilibration period of 10–15 min. at 30°. The extract (0·2–0·5 ml., containing material from 5·0–12·5 mg. dry wt. cells) oxidized nicotinic acid (10–15 μmoles) at the rate of 10–30 μmoles O_2/hr. Oxygen uptake in concentrations of nicotinic acid from $2{\cdot}5 \times 10^{-2}$ to 5×10^{-2} M proceeded linearly for at least 80 min.; at lower concentrations the rate of oxygen uptake was linear for shorter periods and fell off progressively until all the added nicotinic acid was oxidized (Fig. 5). As the oxidation proceeded nicotinic acid, as judged by the CNBr reaction, disappeared and material corresponding in

chromatographic and spectroscopic properties to 6-hydroxynicotinic acid appeared. Total oxygen uptake for the complete oxidation of from 2·0 to 50·0 μmoles nicotinic acid was 0·26–0·46 μmole O_2/μmole nicotinic acid; 0·5 μmole O_2 would be the expected uptake for the conversion of nicotinic acid into 6-hydroxynicotinic acid. The extracts did not oxidize 6-hydroxynicotinic acid, 2-hydroxynicotinic acid or α-hydroxypyridine. Similar results were obtained with extracts of strains 221 and 224.

Properties of the enzyme preparations

Extracts retained their activity unchanged when stored at −15° for 14 days but at 2° all enzyme activity was lost in 4–7 days. From 10 to 20 % of the enzyme activity was lost upon incubation at 30° for 10 min., 50 %, at 40° and 100 % at 60°; all enzyme activity was lost upon heating for 2 min. at 80–100°. Dialysis for 16 hr. at 2° against water or various phosphate buffers completely destroyed the enzyme activity. The lost activity was not restored by the addition of boiled intact cells or boiled extract. Diluting the extracts reduced the rate of oxidation much more than expected (Table 3). This suggested that either a cofactor was being diluted out or that the enzyme was unstable in the more dilute solutions as for instance was found with preparations of formic hydrogenlyase (Gilchrist, 1952). Some attempts were made to fractionate the extracts by centrifuging at 0° for 45 min. and at 10 000 *g*. Three fractions separated as follows: (*a*) partially clarified orange-yellow supernatant containing about 50 % of the original activity, (*b*) an easily disturbed pink deposit with slightly less activity than (*a*), (*c*) a grey deposit which was without activity either alone or when added to (*a*). The addition of boiled extract or boiled intact cells to (*b*) increased the activity from 20 to 40 %. No similar increase resulted from the addition of the boiled material to (*a*). Further centrifuging of (*b*) after dilution with various buffers and salt solutions resulted in the complete and irreversible loss of enzyme activity. It was not possible to clarify the supernatant by further centrifuging up to 60 min. at 20 000 *g*, although there was some gradation in colour from yellow at the top of the tube to pink at the bottom. The extract thus appears to consist of a yellow liquid fraction in which there is suspended a finely divided pink particulate fraction. Removal of the particles reduced the activity of the extract, but it was not possible to recover this lost activity in the washed particulate fraction.

Table 3. *The effect of dilution on the oxidation of nicotinic acid in cell-free extracts*

The extract was prepared from crushed cells as described in the text and the rate of oxidation of nicotinic acid (50 μmoles) followed manometrically.

Extract	Rate of oxygen uptake (μmoles/30 min.)
Undiluted	9·7
Diluted 1:2	1·9
Diluted 1:4	0·4

DISCUSSION

During the oxidation of nicotinic acid by *Ps. fluorescens* the molar ratio between oxygen consumed and nicotinic acid consumed gradually increases from 0·7 to 4 and led previous workers (Pinsky & Michaelis, 1952) to conclude that the first stage in the degradation requires less than 1 μmole of oxygen. These workers also suggested that this first step might be concerned with a reaction involving the α-carbon atom. The present finding that 6-hydroxynicotinic acid may be isolated during the reaction and that cells adapted to nicotinic acid will oxidize the hydroxy derivative fits quite well with the previous data but means that the pyridine ring is first hydroxylated before splitting occurs. The suggestion (Pinsky & Michaelis, 1952) that the carboxyl group remains intact until ring-opening has taken place is thus correct. The oxidative opening of the pyridine ring by a prior hydroxylation reaction thus parallels similar reactions in the benzoic acid series (Happold, 1950) where however the ring-opening reaction is preceded by the formation of diphenols, followed by a split between the two adjacent hydroxyl groups with the formation of derivatives of *cis-cis*-muconic acid (Evans *et al.* 1951). Because 5-fluoronicotinic acid is readily oxidized, the formation of 5:6-dihydroxynicotinic acid would not be a likely further step in the oxidative attack. The possibility of the formation of 2:6-dihydroxynicotinic acid is also unlikely as this compound is not oxidized. It is feasible that because the 6-hydroxy derivative exists in the pyridone form under the conditions of the experiments (Elderfield, 1951) the ring may split directly without further hydroxylation. An analogous opening of the pyridine ring may occur by reaction with cyanogen bromide to give derivatives of glutaconic dialdehyde (cf. Trim, 1948). Such compounds have typical absorption spectra in the 350 mμ. region of the spectrum (Larsen & Haag, 1944; Hughes & Williamson, 1953), but it has not been possible to detect such material during the oxidation of nicotinic acid or 6-hydroxynicotinic acid. It has been possible to confirm the previous findings that ammonia is formed early in the oxidative attack (Pinsky & Michaelis, 1952). The formation of a glutaconic acid derivative therefore seems feasible.

SUMMARY

1. Washed suspensions of *Pseudomonas fluorescens* grown on media containing nicotinic acid oxidize nicotinic acid, 5-fluoronicotinic acid and 2-fluoronicotinic acid at approximately the same rate; 5-chloronicotinic acid is oxidized at a slower rate whilst neither 5-bromonicotinic acid nor 6-fluoronicotinic acid are oxidized.

2. No intermediates in the oxidation were detected when nicotinic acid or the 5-fluoro and 2-fluoro analogues were substrates. An intermediate was detected during the oxidation of 5-chloronicotinic acid; chromatographic and spectroscopic evidence suggested that the pyridine ring was still intact and that substitution of the α-carbon atom had occurred.

3. Washed suspensions of cells grown with asparagine oxidize nicotinic acid after a lag period of 90–120 min. During the later stages of the lag period, 6-hydroxynicotinic acid was isolated and identified by mixed melting point, chromatographic and spectrographic properties.

4. Cells grown on nicotinic acid oxidize 6-hydroxynicotinic acid without a lag period; 2-hydroxynicotinic acid and α-hydroxy pyridine are not oxidized. It is suggested that the formation of 6-hydroxynicotinic acid is the first step in the oxidative attack.

5. Cells grown on asparagine adapt to oxidize 6-hydroxynicotinic acid or 5-fluoronicotinic acid. They do not adapt to the other halogen analogues nor to 2-hydroxynicotinic acid, 2:6-dihydroxynicotinic acid or α-hydroxy pyridine.

6. Extracts prepared from cells broken in a bacterial press converted nicotinic acid into 6-hydroxynicotinic acid; 6-hydroxynicotinic acid was not oxidized.

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Catalase, Peroxidase and Metmyoglobin as Catalysts of Coupled Peroxidatic Reactions

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According to Chodat & Bach (1903) an oxidase is a system composed of oxygenase, an autoxidizable component which on reaction with oxygen produces a peroxide, and peroxidase, which uses this peroxide for oxidation of different substances. This concept, which they believed to be supported by their experiments on fractionation and reconstruction of *Lactarius* oxidase, would in their view explain both the function of peroxidase and the mechanism of action of oxidases. The concept of an oxygenase–peroxidase system was further developed by Thurlow (1925), who grouped under Chodat & Bach's term oxygenase, autoxidizable substances such as cysteine or glutathione and oxidizing